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In Vitro Activity of NifL, a Signal Transduction Protein for Biological Nitrogen Fixation

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In the free-living diazotroph *Klebsiella pneumoniae*, the NifA protein is required for transcription of all *nif* (nitrogen fixation) operons except the regulatory *nifLA* operon itself. NifA activates transcription of *nif* operons by the alternative holoenzyme form of RNA polymerase, σ^{54} holoenzyme. In vivo, NifL is known to antagonize the action of NifA in the presence of molecular oxygen or combined nitrogen. We now demonstrate inhibition by NifL in vitro in both a coupled transcription-translation system and a purified transcription system. Crude cell extracts containing NifL inhibit NifA activity in the coupled system, as does NifL that has been solubilized with urea and allowed to refold. Inhibition is specific to NifA in that it does not affect activation by NtrC, a transcriptional activator homologous to NifA, or transcription by σ^{70} holoenzyme. Renatured NifL also inhibits transcriptional activation by a maltose-binding protein fusion to NifA in a purified transcription system, indicating that no protein factor other than NifL is required. Since inhibition in the purified system persists anaerobically, our NifL preparation does not sense molecular oxygen directly.

In *Klebsiella pneumoniae*, transcription of the nitrogen fixation (*nif*) genes (except *nifLA*) is regulated by the products of the *nifLA* operon. NifA is the transcriptional activator required for expression of the *nif* genes, and NifL is a negative regulator that inhibits NifA activity in response to molecular oxygen or combined nitrogen (1, 5, 10, 13, 22). The *nif* genes are transcribed by an alternative holoenzyme form of RNA polymerase, σ^{54} holoenzyme, and NifA catalyzes isomerization of closed complexes between this polymerase and a promoter to transcriptionally productive open complexes (Fig. 1) (3, 24 [reviewed in reference 18]). To do so, NifA binds to an upstream activation sequence that is typically located more than 100 bp upstream of the transcriptional start site and contacts promoter-bound RNA polymerase by means of a DNA loop that is facilitated by the integration host factor (IHF) (15, 23, 31). We have purified a maltose-binding protein fusion to NifA (MBP-NifA) and used it to demonstrate DNA binding and activation of transcription by NifA in vitro (3, 18, 19). In a purified system, catalysis of open complex formation (transcriptional activation) depends upon a nucleoside triphosphate with a hydrolyzable β-γ bond. Although we infer that NifA must have nucleoside triphosphatase activity, we were unable to demonstrate such activity for MBP-NifA because our preparations were contaminated by other nucleoside triphosphatases that we could not remove (18a).

NifA from *K. pneumoniae* is composed of three domains: a central catalytic domain, a carboxy-terminal DNA-binding domain, and an amino-terminal regulatory domain (6, 7, 25). The central domain, which is directly responsible for transcriptional activation, is homologous to that of NtrC, the best-characterized σ^{54}-dependent activator. The N-terminal domain of NifA, which has no known homolog, appears to modulate sensitivity to NifL (8).

NifL from *K. pneumoniae* is a 495-amino-acid protein that is composed of two domains separated by a flexible glutamine-rich linker (9). Strikingly, a portion of its amino-terminal domain shows homology (30% amino acid identities over ~124 residues) to the product of the *bat* gene from *Halobacterium halobium* (36), which is required for expression of bacterio-opsin (21). (The same homology is observed for the NifL protein from *Azotobacter vinelandii* [4, 29].) Since expression of bacterio-opsin is known to be oxygen sensitive (32, 36), as is the function of NifL, it has been proposed that the region of homology between them may be involved in oxygen sensing (4, 36). A portion of the carboxy-terminal domain of NifL (~50 amino acids) shows amino acid sequence similarity to region 1 of autokinases (sensors) of two-component regulatory systems but lacks the histidine residue that is normally phosphorylated (4, 9). The mechanism(s) by which NifL senses oxygen and combined nitrogen is not known.

In vivo, NifL inhibits DNA-binding and transcriptional activation by NifA (26), and there is evidence that inhibition is mediated by stoichiometric interaction between the two proteins (11). Congruent with the latter evidence, it was previously reported that S30 extracts prepared from a strain that overproduced both NifL and NifA failed to show NifA activity in a coupled transcription-translation assay (2). In this report, we demonstrate NifL inhibition of NifA activity in vitro in both a coupled transcription-translation system and a purified transcription system. Inhibition is specific for NifA but is not sensitive to the presence or absence of molecular oxygen or the NH_4^+ ion.

MATERIALS AND METHODS

Plasmids. Plasmid pJES282, which was derived from pDOJ03 (28), is a pT7-7-based vector that carries the *nifLA* operon (from the ATG encoding the first methionine of NifL to the *Acc*I restriction site downstream of *nifA*). Plasmids pJES294 (30) and pJES468 (19) carry *nifA*, and pJES283 carries *nifL*. Plasmid pJES283 was constructed as follows. An *Xba*I-*Dde*I (*Dde*I end made blunt with the Klenow fragment of DNA polymerase I) fragment of pJES282 carrying the T7 ribosome binding site, *nifL*, and ~100 bp of *nifA* was ligated into pT7-7 that had been digested with *Xba*I and *Hin*dIII

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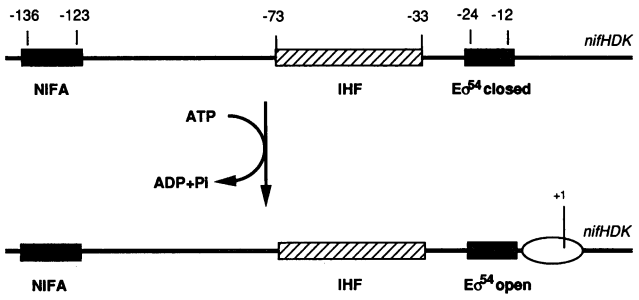

FIG. 1. Formation of open complexes at the *nifH* promoter of *K. pneumoniae*. The upstream NifA-binding site, region of protection by IHF, and E · σ^{54}-dependent promoter are indicated by boxes. With respect to the transcriptional start site at +1, the conserved TGT and ACA motifs in the NifA half sites are centered at positions −136 and −123, respectively. Boundaries of protection from DNase I digestion by IHF lie at approximately positions −73 and −33. Conserved GG and GC doublets in the promoter are at positions −24 and −12, respectively. Formation of open complexes requires a nucleoside triphosphate with a hydrolyzable β-γ bond.

(*Hin*dIII end made blunt with the Klenow fragment). Plasmid pJES597 encodes a fusion of MBP to the N-terminal methionine of NifA (19). A description of the construction of plasmid pJES559, which encodes an MBP fusion to NtrC, is being prepared (17). Plasmids pJES384 (31), p318 (30), and pJES128 (deletion derivative of pJES142 [p*nifH*] described in references 19 and 35) carry the *nifH* promoter-regulatory region of *K. pneumoniae*. Plasmids pJES384 and p318 carry a translational fusion of *nifH* to *lacZ*, and p318 carries a mutation in the *nifH* promoter that increases its affinity for E · σ^{54} in closed complexes (31). Plasmids pJES399 and pJES409 carry as their only activator-binding site an NtrC site in place of the NifA site upstream of the *nifH* promoter. Plasmid pJES399 carries a translational fusion of *nifH* to *lacZ*. Plasmid pJES40 carries the *glnA* promoter-regulatory region and a translational fusion of *glnA* to *lacZ* (14), and plasmid pRS229 carries *lacZ* under control of the *lacUV5* promoter (33).

Cell extracts containing NifL and NifA. Crude supernatants containing NifL or NifA were prepared from *Escherichia coli* NCM632 carrying pJES283 or pJES294, respectively (19). Even though NifL and NifA were insoluble when overproduced, a small amount of each remained in the crude supernatant and was active (see Results [2, 15, 34]). A control crude supernatant lacking NifL was prepared from NCM632 carrying plasmid pJES307, which is a pT7-7-based vector with no cloned gene.

Purification of NifL and other proteins. NifL was purified as follows. Crude pellet (as opposed to the crude supernatant prepared as described in reference 19) obtained from 0.5 liter of culture of *E. coli* NCM632 carrying plasmid pJES283 was resuspended in 30 ml of breakage buffer {20 mM potassium EPPS (*N*-[2-hydroxyethyl]piperazine-*N'*-3-propanesulfonic acid, pH 8.0), 125 mM potassium glutamate, 5% (vol/vol) glycerol, 1.5 mM dithiothreitol} (15) containing EDTA (1 mM), lysozyme (6.3 mg [Sigma]), and deoxycholate (30 mg). After a 30-min incubation at room temperature, the suspension was subjected to centrifugation at 20,000 × *g* for 30 min. The resulting pellet was resuspended in 25 ml of EPPS buffer (10 mM [pH 8.0]) containing urea (8 M), incubated for 30 min at room temperature to solubilize NifL, and subjected to centrifugation at 20,000 × *g* for 30 min. To renature NifL, the supernatant was dialyzed against breakage buffer containing 25 mM potassium glutamate instead of the usual 125 mM (4 liters) at 4°C for 4 h. Dialysis was continued overnight against fresh buffer (2 liters). After dialysis, insoluble material was removed from the NifL preparation by centrifugation at 20,000 × *g* for 30 min. A portion of the supernatant (10 ml/25 ml total) containing renatured NifL (2.3 mg of protein per ml) was applied to a Sephacryl S-200HR column (2.6-cm inner diameter by 50-cm height of resin) that had been equilibrated with the dialysis buffer described above, and fractions containing NifL were quick-frozen in small portions, saved separately, and used only once. For most experiments, the peak fraction was used. Although other fractions were active, they were dilute. The concentration of NifL was calculated with the assumption that it is a monomer because it eluted from the S-200 column in an undefined oligomeric state (see Results).

MBP-NifA was purified as previously described (19), and its concentration was calculated with the assumption that it is a dimer. A description of the purification of MBP-NtrC is being prepared (17).

Coupled transcription-translation and transcription assays. Coupled transcription-translation assays and transcription assays were performed as previously described (19, 20). S30 extract was prepared from *Salmonella typhimurium* SK419. In addition to MBP-NifA (or MBP-NtrC), NifL, and the appropriate template (5 nM), transcription reaction mixtures contained E · σ^{54} (60 nM core polymerase, 100 nM σ^{54}), IHF (60 nM), and NtrB (50 nM), which was present only when NtrC or MBP-NtrC was the activator. Open complex formation was initiated by addition of ATP (to a final concentration of 1.6 mM), and open complexes were subsequently detected with a single-cycle transcription assay.

For transcription assays performed anaerobically, ammonium acetate was omitted from the buffer. Air was removed from components (buffer containing σ^{54} holoenzyme, IHF, and template; MBP-NifA; NifL; ATP; heparin) by 20 cycles of flushing with N_2 gas (~4 lb/in^2, 5 s) followed by degassing on a vacuum pump for 1 min (10 cycles of flushing were sufficient to make vials anaerobic as assessed with methylene blue indicator strips [BBL GasPak disposable anaerobic indicator]). Components were then mixed by using a Hamilton syringe that had been rinsed with deaerated water. NifL was added to the other components first, followed by MBP-NifA. After addition of MBP-NifA, the reaction mixture was incubated at 30°C for 10 min. Formation of open complexes was then initiated by adding ATP. After an additional 10 min at 30°C, heparin was added to stop formation of further open complexes. Five minutes after heparin was added, vials were opened, and after 5 min, the remaining nucleotides were added. Transcript synthesis was allowed to proceed for 10 min. Monitoring with methylene blue strips indicated that anaerobic conditions were maintained until vials were opened, i.e., throughout the period allowed for open complex formation. Control experiments were carried out aerobically with the same vials and syringes used for anaerobic assays.

RESULTS

NifL synthesized in a coupled transcription-translation system inhibits the activity of NifA similarly synthesized. To initiate studies of NifL, we directed synthesis of the NifL and NifA proteins in a coupled transcription-translation system and monitored the effect of NifL on NifA activity. Synthesis of NifL and/or NifA was directed in one reaction mixture, and then NifA activity was monitored in a second reaction mixture with a reporter plasmid carrying a fusion of the *nifH* promoter-regulatory region to *lacZ*. When NifA alone was synthesized, it

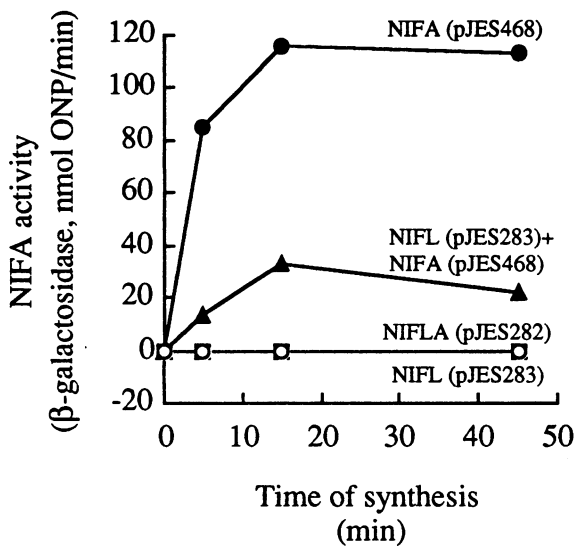

FIG. 2. Inhibition of NifA activity by NifL when both are synthesized in a coupled transcription-translation system. Assays in the coupled system were performed as described previously (30). All incubations were at 30°C. NifL and/or NifA were synthesized in vitro from driver plasmids (described below [20 nM = 5 μg]). Synthesis of these proteins was started with T7 RNA polymerase (40 U/50 μl of reaction mixture), and then samples (6 μl) were taken at the times indicated and assayed for NifA activity with plasmid p318 (5 μg) as reporter. Assay tubes contained reporter, S30 extract, and σ^{54} (110 nM). After 2 h of incubation, β-galactosidase activity, which reflects NifA activity, was measured as previously described. Driver plasmids were pJES468, which carries *nifA* alone; pJES283, which carries *nifL* alone; the combination of pJES468 plus pJES283; and pJES282, which carries *nifL* and *nifA* in their natural configuration. We do not know why the loss of NifA activity observed after 20 to 30 min in previous experiments (30) is not apparent here. ONP, *o*-nitrophenol.

activated transcription from the reporter plasmid (Fig. 2, ●). When NifL and NifA were synthesized from a single driver plasmid carrying the *nifLA* operon (i.e., with *nifL* and *nifA* in their natural configuration), no trace of NifA activity was detected (Fig. 2, ○). When the two proteins were synthesized from separate plasmids carrying the *nifL* and *nifA* genes, NifA activity was inhibited ~80% by NifL (Fig. 2, ▲), providing evidence that translational coupling between *nifL* and *nifA* is not absolutely required for NifL inhibition (11).

NifL in crude cell extracts inhibits NifA activity in the coupled system. To study the inhibitory effects of NifL synthesized in vivo on NifA activity in the coupled system, we provided NifA from a crude supernatant of a strain that overproduced it and determined the effect of adding NifL from a similar crude supernatant on activation of transcription by NifA. As is true for NifA, NifL forms insoluble inclusion bodies when it is overproduced in *E. coli* (2). Even though crude supernatants obtained from cell extracts of such overproducing strains after low-speed centrifugation (19) contained very little NifL (as judged by Coomassie blue staining of sodium dodecyl sulfate [SDS]-polyacrylamide gels), they contained NifA-inhibiting activity (Fig. 3A, ●). (Crude supernatants of NifA-overproducing strains also contain very little NifA [2, 15, 34].) When equal volumes (5 μl) of crude supernatants containing NifL and NifA were used, NifA activity was inhibited ~75%. The inhibitory activity was specific to NifA, as judged by the fact that it did not affect NtrC, a homolog of NifA (Fig. 3B, ■). In the experiment shown, NtrC-dependent activation was measured from the *nifH* promoter with a template in which the NifA-binding site had been precisely replaced with an NtrC site. NifL also failed to inhibit NtrC-dependent activation from the *glnA* promoter (plasmid pJES40 as template [data not shown]). Similarly, expression from the σ^{70}-dependent *lacUV5* promoter was little affected by NifL (Fig. 3C, ▲).

To demonstrate in an independent way that inhibition of NifA activity by crude supernatants containing NifL was specific, we tested the effect of a control supernatant prepared from a strain carrying the vector used for NifL overexpression (Fig. 3A, B, and C, open symbols). The control supernatant appeared to stimulate NifA activity (Fig. 3A, ○).

Renatured NifL inhibits NifA in the coupled system. We purified NifL from inclusion bodies by denaturing it in urea, removing the urea by dialysis, and purifying the renatured material by gel filtration chromatography as described in Materials and Methods. NifL eluted in the void volume of a Sephacryl S-200HR column, indicating that it was aggregated (not shown). The resulting NifL contained only a few minor contaminants, as assessed by Coomassie blue staining of SDS-polyacrylamide gels (Fig. 4).

Renatured NifL inhibited the activity of NifA from crude supernatants in the coupled system (Fig. 3D) and also inhibited the activity of MBP-NifA (not shown [see below]). The inhibition was specific to NifA because renatured NifL did not inhibit NtrC-activated transcription from the *nifH* promoter (Fig. 3E) or σ^{70}-dependent transcription from the *lacUV5* promoter (Fig. 3F). However, renatured NifL was less effective in inhibiting NifA activity than crude supernatants prepared from the NifL-overproducing strain; much larger amounts of the renatured protein were required.

We attempted to improve the activity of renatured NifL in several ways. (i) NifL was dialyzed away from urea at protein concentrations of between 0.07 and 0.3 mg/ml. (ii) The pH of the buffer used for dialysis was changed from 8 to 7 or 9. (iii) The glycerol concentration in the buffer was increased from 5% to 20% (vol/vol). (iv) Dithiothreitol was omitted. (v) EDTA (1 mM) or $MgSO_4$ (1 mM) was added. (vi) $FeCl_3$ or $FeSO_4$ (0.1 mM) was added, and dithiothreitol was omitted. None of these changes improved inhibition by renatured NifL, as assessed in the coupled transcription-translation system (not shown).

NifL inhibits the activity of MBP-NifA in a purified transcription system. Since purified NifA is insoluble and inactive, we have studied NifA activity in a purified transcription system with MBP-NifA (19). Renatured NifL inhibited the activity of MBP-NifA in the purified system (Fig. 5, ● [0.74 μM MBP-NifA]). Inhibition was 90% at 2 μM NifL (2.7-fold molar excess of NifL over NifA, assuming NifL is a monomer, or 1.4-fold molar excess, assuming NifL is a dimer). Similar inhibition was observed when GTP or UTP was used in place of ATP (not shown) as the nucleotide to allow open complex formation. Even at concentrations as high as 6.7 μM (monomer), renatured NifL had no inhibitory effect on the activity of MBP-NtrC (40 nM). As was the case for MBP-NifA, the amount of MBP-NtrC used for this experiment was chosen to be subsaturating so that any possible inhibition by NifL would be detected with maximum sensitivity. Renatured NifL did not affect transcriptional activation by MBP-NtrC even when lower concentrations (5 or 10 nM) were used (data not shown).

We also studied NifL inhibition of MBP-NifA activity under anaerobic conditions (ammonium acetate was omitted from the transcription buffer [see Materials and Methods]). Inhibition was unchanged (not shown).

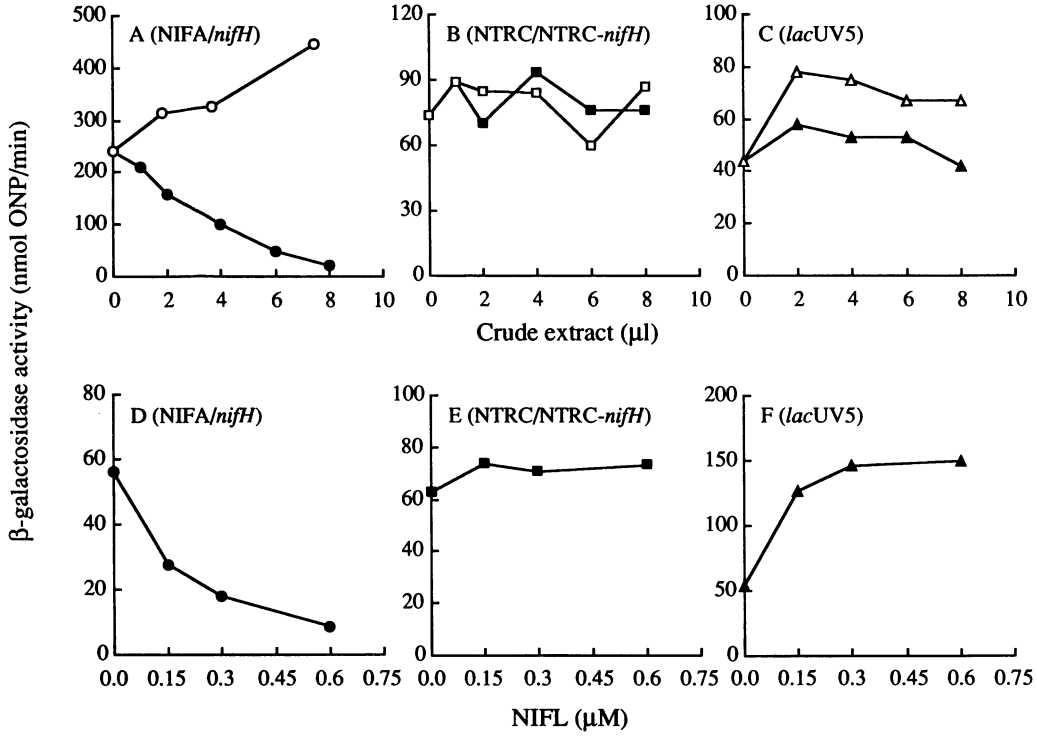

FIG. 3. Inhibition of NifA activity by NifL in crude supernatants (A to C) and after renaturation from urea (D to F). Assays were performed with a coupled transcription-translation system at 30°C in a total volume of 50 μl as described previously (19). NifL was provided from a crude supernatant of the overproducing strain NCM632/pJES283 (~6.5 mg of protein per ml [A to C]) or after extraction from inclusion bodies and renaturation (D to F [see Materials and Methods; concentrations were calculated with the assumption that NifL is a monomer). NifL was added to the other components of the reaction mixture before NifA or NtrC. NifL activity was detected by inhibition of NifA activity (● [A and D]); the source of NifA was a crude supernatant (5 μl [~32 μg of total protein]) prepared from the overproducing strain NCM632/pJES294, and NifA activity was monitored at the *nifH* promoter with plasmid pJES384 (5 μg) as the reporter. σ^{54} was added to 110 nM. The activity of purified NtrC (88 nM [■; B and E]) was monitored at the *nifH* promoter with plasmid pJES399 (6 μg), which carries an NtrC site in place of the NifA site upstream of *nifH*, as the reporter. NtrB was added to 47 nM, and σ^{54} was added to 110 nM. The activity of σ^{70} holoenzyme present in the S30 extract (▲ [C and F]) was monitored at the *lacUV5* promoter with plasmid pRS229 (5 μg) as the reporter. (No σ^{54} was added to the extract in this case.) The amounts of NifA, NtrC, and *lacUV5* plasmid used were not in excess, so that NifL inhibition would be maximal. Crude supernatant lacking NifL (prepared from strain NCM632/pJES307 [~6.5 mg of protein per ml]) was used as a control for NifL inhibition in panels A to C (open symbols). ONP, *o*-nitrophenol.

DISCUSSION

In vivo, NifL antagonizes the action of NifA in response to molecular oxygen or combined nitrogen. As a first step toward understanding the signal transduction that controls NifL function and the means by which NifL inhibits NifA activity, we established assays for NifL in vitro. NifL specifically inhibits NifA and MBP-NifA activity in a coupled transcription-translation system and inhibits MBP-NifA activity in a purified transcription system. It does not inhibit the activity of NtrC, a homolog of NifA, in either system, nor does it inhibit transcription by σ^{70} holoenzyme.

We were able to demonstrate NifL inhibition of NifA activity with three different types of NifL preparations. First, we showed the inhibitory effect of NifL that was synthesized in a coupled transcription-translation system. Second, we showed the inhibitory effect of NifL that was present in crude supernatants prepared from an NifL-overproducing strain. As was the case for NifA, crude supernatants from the overproducing strain had NifL activity even though they contained very little NifL protein; most of the overproduced NifL was insoluble. Finally, we could demonstrate the activity of NifL that was extracted from inclusion bodies with urea and renatured. This material inhibited the activity of MBP-NifA in a purified

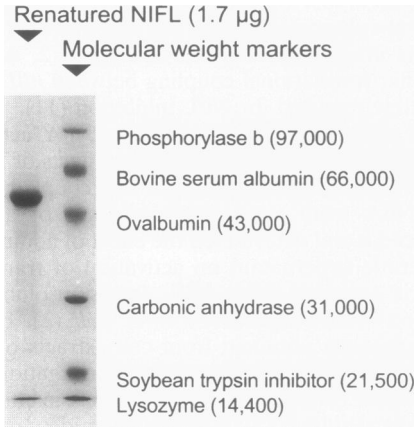

FIG. 4. Purity of renatured NifL. The purity of renatured NifL was assessed by Coomassie blue staining of an SDS-polyacrylamide gel (12% polyacrylamide). NifL was partially purified from inclusion bodies as described in Materials and Methods. Lanes: 1, 1.7 μg of NifL after chromatography on Sephacryl S-200HR; 2, molecular weight markers (low range; Bio-Rad).

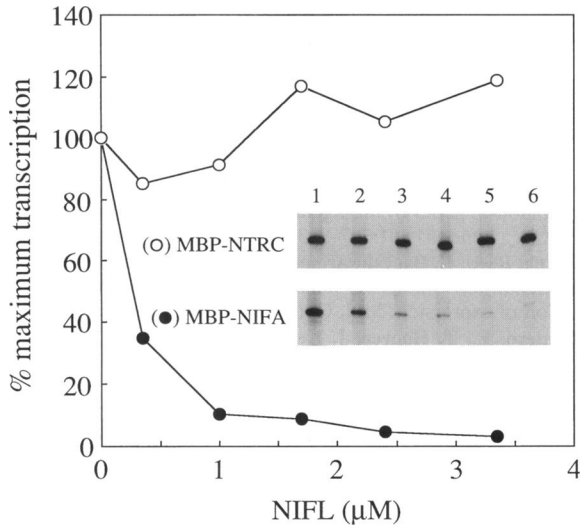

FIG. 5. Inhibition of MBP-NifA activity by NifL in a purified transcription system. The activity of MBP-NifA (0.74 μM with the assumption that it is a dimer) was assessed at the *nifH* promoter with plasmid pJES128 as template, and the activity of MBP-NtrC (40 nM dimer) was assessed at the same promoter with plasmid pJES409, in which an NtrC site replaces the upstream NifA site, as template. NtrB (50 nM) was present for reaction mixtures containing MBP-NtrC. To assess NifL inhibition, NifL was added to the other reaction mixture components prior to NifA or MBP-NtrC. After addition of the latter proteins, formation of open complexes was initiated by addition of ATP and open complexes were detected by their ability to yield transcripts in a single-cycle transcription assay. Both MBP-NifA and MBP-NtrC were subsaturating (see Results) to allow ready detection of NifL inhibition. The amount of transcript obtained with MBP-NifA alone (100%) was 2.8 fmol and that obtained with MBP-NtrC alone was 3.6 fmol. The inset shows an autoradiogram of transcripts. Concentrations of NifL (micromolar monomer) were 0, 0.7, 2.0, 3.4, 4.8, and 6.7 in lanes 1 to 6, respectively. Radioactivity in transcript bands was quantitated with a Molecular Dynamics Phosphorimager and is plotted as the percentage of maximum transcription.

transcription system, indicating that no additional protein factor or small molecule (but see below) was necessary for NifL inhibition. Renatured NifL had little activity with respect to the small amount of NifL that remained in crude supernatants; i.e., high concentrations of renatured NifL were required to fully inhibit NifA activity in the coupled system (compare Fig. 3D and 3A). Initial efforts to improve renaturation, which included adding ferrous or ferric iron to the dialysis buffer, were unsuccessful (see Results). (The presence of iron or manganese in the growth medium was reported to be required for NifL activity in vivo [11].)

Our observations are congruent with a proposed mechanism in which NifL inhibition depends upon stoichiometric interaction with NifA (2, 11) rather than covalent modification of NifA. If, in fact, covalent modification is involved, ATP (or GTP or UTP) is presumably the donor since one of these nucleotides is the only small molecule present during NifL inhibition of open complex formation by MBP-NifA in a purified transcription system. Although the carboxy-terminal domain of NifL has limited sequence similarity to autokinases (sensors) of two-component regulatory systems (9), we detect no incorporation of phosphate from [γ-^{32}P]ATP into NifL or into MBP-NifA under conditions in which the function of the latter is inhibited (27). Moreover, other autokinases are quite specific for ATP (12, 16), whereas NifL inhibits NifA activity when ATP is replaced with GTP or UTP as the nucleotide for open complex formation.

In the purified transcription system, NifL was competent to inhibit NifA activity in the absence of molecular oxygen or a physiological source of combined nitrogen, indicating that renatured NifL does not sense these small molecules directly. Since the NifL we employed was isolated from cells that had been grown aerobically and under conditions of nitrogen excess, we think it likely that NifL was either modified in vivo in response to one or both of these signals (presumably covalently) or is inhibitory (functional) in its unmodified form. In the latter case, some sort of alteration or signalling would be needed to convert NifL to a noninhibitory (nonfunctional) form when both oxygen and combined nitrogen are limiting.

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